THE USE OF ALPHA LIPOIC ACID IN THE ANTIMETASTATIC TREATMENT

The invention relates to the use of alpha lipoic acid, also known as lipoic acid, thioctic acid or 1,2-dithiolan-3-pentanoic acid, as well as derivatives thereof, in the control of tumour progression and in the antimetastatic therapy.

TECHNOLOGICAL BACKGROUND

It is universally accepted that cancerogenesis is a multiphase process in which at least three development phases are recognised: initiation, promotion and progression (Rous and Kidd, J. Exp. Med., 73: 365-376, 1941; Beremblum and Shubik, Br. J. Cancer 1: 383-386, 1947; Foulds L., Cancer Res., 14: 327-339, 1954). In the progression phase, a cell population is selected which lacks control of proliferation and acquires malignant characteristics, giving rise to the metastatic process. The diagnosis and treatment of tumours usually begin at a late stage when most patients already have occult or overt metastasis. In particular the critical pathological turning point is the initiation of local invasion leading to the dissemination of tumour cells. An important window of therapeutical intervention can be defined as the period during which transition from hyperproliferative state to the acquisition of the capacity for invasion and metastasis occurs (Kohn and Liotta, Cancer Res., 55: 1856-1862, 1995). Treatment with an antimetastatic agent can delay or block the processes of invasion and metastasis, increasing the chance of survival. These drugs should be administered daily and for long-term therapies.

Most recently identified antimetastatic and/or tumour progression inhibiting agents recently found (BB2516 Marimastat, BB94 Batimastat, BB3644 (British Biotech),

BAY129566 (Bayer), AG3340 (Agouron), CGS27023A (Novartis), RO32-3555 (Roche), D2163, D5410 (Chiroscience), Metastat (CollaGenex) are synthetic derivatives and have high pharmacological effectiveness. These compounds share an inhibiting activity on metalloproteinases, which participate in degradation of the basal membrane. Unfortunately, therapeutical doses often involve adverse toxic effects (musculo-skeletal, hepatic and gastric toxicities), which prevent long-term treatments as well as repeated daily administrations, although making it possible their use in therapy courses. Furthermore, therapy courses are particularly expensive. Another class of antimetastatic agents is possibly represented by natural polypeptides (TIMPs) (Albini, Pathol. Oncol. Res. 4, 3: 230-241,1998). Obviously, these molecules cannot be administered through the oral route, have low membrane permeability and high costs.

It has been reported that alpha lipoic acid, a known antioxidant molecule used in clinical practice as a therapeutic agent for liver disorders, can be used in different pathologies such as arthritis, ulcer, HIV infection (EP 427287). Alpha lipoic acid is a natural compound, with poor or no adverse effects even at high dosages in humans. Alpha lipoic acid esters were claimed as antineoplastic (CH 683,920) and antitumoral (DE 4400843) agents.

The capability of lipoic acid of inhibiting the malignant transformation of cell lines was described by Colacci et al., and by Silingardi et al., respectively at the 88th Annual Meeting of the American Association for Cancer Research, San Diego, California, USA, April 12-16, 1997, and at the 89th Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana, USA, March 28- April 1, 1998.

On the other hand, such potential chemopreventive effect does not allow to draw conclusions about any potential antimetastatic effect of lipoic acid. This effect does not depend on cytotoxic or cytostatic mechanisms, but rather involves the inhibition of cell migration, adhesion and invasion.

DEFINITION OF THE USED WORDS

The following definitions will be used in the disclosure of the invention.

Invasiveness: Ability of cells to cross anatomic barriers, such as basal membranes, interstitial stroma and intercellular junctions which divide tissue compartments (Mignatti and Rifkin, Physiol. Rev., 73: 161-195, 1993).

Migration: one of the steps of invasion, motility, which allows tumour cells to cross basal membrane and stroma (Liotta et al., Sem. Cancer Biol., 2: 111-114, 1991).

Chemoinvasion: Invasive response of the cells to a chemoattractant stimulus.

Chemoattractant: mixture of substances of cellular derivation capable of stimulating directional migration.

Adhesion: ability of the cells to specifically recognise and attach to extra-cellular matrix.

DISCLOSURE OF THE INVENTION

It has surprisingly been found that alpha lipoic acid (LA) or the salts thereof have a high antimetastatic activity at micromolar doses; lipoic acid inhibits chemoinvasion and causes an increase in tumour cell adhesion to the extra-cellular matrix. Alpha lipoic acid can be used either as the racemate or in the enantiomerically pure form.

The antimetastatic activity of lipoic acid was demonstrated by using a chemoinvasion model (Albini et al., Cancer Res., 47: 3239-3245, 1987; Reich et al., In:

"Alternative Methods in Toxicology, Goldberg and Liebert eds., Vol. 7, pp 11-22, 1989), which allows a rapid, quantitative and reproducible assessment of the invasive and metastatic potential of malignant cells and therefore a reliable identification of molecules with antimetastatic activity. In vitro models mimicking the invasion process are effective screening tools to detect for detecting compounds with antiinvasive and antimetastatic activities (Hart and Fidler, Cancer Res., 38: 3218-3224, 1978; Liotta et al., Cancer Lett., 11:141-147, 1980; Starkey et al., Cancer Res. 44: 1585-1594, 1984, Mareel et al., Inv. Met. 1: 195-204, 1981). Further evidences of the antimetastatic activity of lipoic acid are provided by its high ability to promote cellular adhesion to the basal membrane. Again, a standard in vitro protocol was used (Kato and De Luca, Exp. Cell Research 173, 450-462, 1987; Kato et al., Exp. Cell Research 179, 31-41, 1988; Kim et al., Inv. Met 14, 1-6: 147-155, 1994-1995).

EXPERIMENTAL SECTION

The cell lines used in this test show a fully malignant phenotype: murine fibroblasts (BALB/c 3T3) transformed with carcinogenic agents: 1,2-dibromoethane (clone F4), 3-methylcholanthrene (clone MCA1), benzo(a)pyrene (B(a)P); murine fibroblasts (NIH3T3) transfected with H-ras (NIH/ras), and the human fibrosarcoma cell line HT1080.

CHEMOINVASION ASSAY

The chemoinvasion assay was performed according to the standard procedure (Albini et al., Cancer Res., 47: 3239-3245, 1987; Melchiori et al., Inv. Met., 12, 1-12, 1992, Adatia et al., Inv. Met., 13: 234-243, 1993; Albini, Pathol. Oncol. Res. 4, 3: 230-241, 1998) using the artificial basal membrane Matrigel(R). In the chemoinvasion assay, normal fibroblasts and epithelial cells, as well as

cells deriving from benign tumours, cannot cross the Matrigel(R) coating. Malignant cells, having specific basal membrane degrading enzymes, penetrate the gel and migrate to the lower surface of the filter after 6 hour incubation. The number of metastatic cells crossing the Matrigel(R) and their malignant behaviour are directly related (Albini et al., Cancer Res. 47: 3239-3245, 1987).

The following Tables 1-3 show the number of cells (per field) which crossed the Matrigel(R) barrier and the percentage of invasion inhibition compared to simultaneously tested controls. The mean of three different experiments in triplicate are reported. A reduction of invasion $\geq$30% is considered to be significant (Welch et al., Int. J. Cancer :43, 449-457, 1989).

Table 1 shows results from the assay performed pre-treating the malignant cells with alpha lipoic acid. 70% confluence cells were treated with an alpha lipoic acid solution (0.1-100 μM) obtained by dissolving the product in 1N NaOH. After 16h the cells were harvested with trypsin-EDTA (0.05% and 0.02%, respectively), resuspended in 10% NCS D-MEM, centrifuged, washed with D-MEM containing bovine serum albumin (BSA, 0.1%), centrifuged again and resuspended in the same medium. The viability and the number of cells were assessed by the trypan blue exclusion test. The invasion assay was performed according to the standard procedure (Albini et al., Cancer Res. 47: 3239-3245, 1987) using a cell suspension containing 1.5×10^5 cells / chemotaxis chamber.

Table 1. Effects of LA pretreatment (16 hrs) on the invasive behaviour of murine cells transformed by chemicals or by oncogene transfection.

	DBE/F4		MCA1		B(a)P		NIH/ras	
LA (μM)	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition
0	116 ± 3		116 ± 2		151 ± 4		174 ± 6	
0.1	78 ± 1	32 ± 1	77 ± 1	39 ± 1	140 ± 1	7 ± 1	134 ± 5	23 ± 1
1	60 ± 1	48 ± 1	60 ± 1	48 ± 1	109 ± 2	28 ± 1	102 ± 2	41 ± 1
10	48 ± 1	28 ± 1	40 ± 1	65 ± 1	86 ± 3	43 ± 2	74 ± 3	57 ± 1
100	42 ± 1	64 ± 1	30 ± 1	74 ± 1	68 ± 1	55 ± 1	62 ± 10	64 ± 6

In table 2 results of an invasion assay performed in the presence of alpha lipoic acid are reported. Exponentially growing cells were harvested with trypsin-EDTA (0.05% and 0.02% respectively), resuspended in 10% NCS D-MEM, centrifuged, washed with D-MEM containing bovine serum albumin (BSA, 0.1%), centrifuged again and resuspended in the same medium containing alpha lipoic acid (0.1-100 μM conc.) previously solubilised in 1N NaOH. The viability and the number of cells were assessed by the trypan blue exclusion assay. The invasion assay was performed according to the standard procedure (Albini et al., Cancer Res. 47: 3239-3245, 1987) using a cell suspension containing 1.5 x 10^5 cells/ chemotaxis chamber (0.8 ml).

Table 2. Effects of LA treatment on the invasive behaviour of murine cells transformed by chemicals or by oncogene transfection.

	DBE/F4		MCA1		BP		NIH/ras	
LA (μM)	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition
0	120 ± 1		102 ± 2		154 ± 2		130 ± 2	
0.1	85 ± 1	29 ± 1	80 ± 1	21 ± 1	105 ± 4	32 ± 1	73 ± 1	44 ± 1
1	78 ± 1	35 ± 1	72 ± 2	40 ± 2	106 ± 1	31 ± 1	67 ± 2	48 ± 2
10	44 ± 1	63 ± 1	47 ± 1	61 ± 1	85 ± 3	45 ± 2	56 ± 1	57 ± 1
100	40 ± 1	67 ± 1	35 ± 1	66 ± 1	72 ± 1	53 ± 1	37 ± 1	71 ± 1

Table 3 shows the results from the invasion assay carried out on HT1080 cells. This cell line was isolated from a human fibrosarcoma and is widely used in cancer research because of its characteristics (high invasive and metastatic behaviour).

70% confluence cells were pre-treated with a solution (0.1-100 μM) of alpha lipoic acid (obtained by dissolving the product in 1N NaOH), or were resuspended after removal in 10% NCS D-MEM medium containing alpha lipoic acid (0.1-100 μM). The assay was carried out according to the standard procedure (Albini et al., Cancer Res. 47: 3239-3245, 1987) using a cell suspension containing 1.5 x 10^5 cells / chemotaxis chamber.

Table 3. Effects of LA on the invasive behaviour of HT1080 cells

	Pretreatment		Simultaneous treatment	
LA (μM)	No. cells ± S.E.	% inhibition	No. cells ± S.E.	% inhibition
0	434 ± 2		438 ± 2	
0.1	391 ± 1	10 ± 1	387 ± 5	12 ± 1
1	323 ± 13	26 ± 3	311 ± 1	29 ± 1
10	311 ± 3	28 ± 1	249 ± 4	43 ± 1
100	198 ± 2	54 ± 1	188 ± 4	57 ± 1

RESULTS OF THE CHEMOINVASION ASSAY

The results clearly demonstrated the anti-invasive dose-related effect of lipoic acid. The compound inhibits the invasive capability of malignant murine cells obtained by chemical transformation (clones MCA1 and DBE/F4) or by transfection with an activated oncogene (NIH/ras). Consistent results are obtained in human fibrosarcoma - derived cells. Compared with the untreated control, a 30% inhibition is observed at dosages ranging from 0.1-1μM. Similar results are observed when dissolving alpha lipoic

acid in KOH, tris(hydroxymethyl)-aminomethane or EtOH. The micromolar activity as well as the lack of toxicity demonstrate that lipoic acid strongly inhibits the invasion process and its effect does not depend on the administration schedule.

ADHESION ASSAY

Cell adhesion, a basic phenomenon for the metastatic process, was tested through of a widely used in vitro model (Kato and De Luca, Exp. Cell Research 173, 450-462, 1987; Kato et al., Exp. Cell Research 179, 31-41, 1988; Kim et al., Inv. Met 14, 1-6: 147-155, 1994-1995). Adhesion to the extracellular matrix plays a pivotal role in assessing the ability of tumour cells to migrate to distant sites, leading to metastasis onset. A high adhesion to the extracellular matrix is linked to a lower tendency to migrate (Wagner et al., Proc. Natl. Acad. Sci. USA 92: 7411-7415, 1981; Varner and Cheresh, Curr. Opin. Cell Biol. 8: 724-730. 1996) and therefore to an antimetastatic effect of the product (Glinsky, Cancer and Met. Rev., 17: 177-185, 1998).

Exponentially growing cells were mechanically removed, resuspended in 0.05% BSA D-MEM and centrifuged twice. The cell number was evaluated by Trypan-blue exclusion assay and then diluted with 0.05% BSA D-MEM containing lipoic acid (100-500 μM) to a density of 2×10^5 cells / ml, 1 ml of cell suspension per plate and incubated for 2 h at 37°C 5% CO_2. Plates were coated according to the procedure previously described (Kato and De Luca, Exp. Cell Research 173, 450-462, 1987; Kato et al., Exp. Cell Research 179, 31-41, 1988; Kim et al., Inv. Met 14, 1-6: 147-155, 1994-1995) using as adhesion substrates fibronectin (3 μg/ml), laminin (10 μg/ml), vitronectin (3 μg/ml max conc.), collagen IV (10 μg/ml). Plates were then washed 3 times with adhesion medium, washed with PBS, fixed and stained in 0.2% crystal violet in 20% methanol for 10 min. The excess

of dye was removed.

Tables 4 - 7 show the optical density (measured at 560 nm wavelength) of the solution obtained by solubilising the dye fixed to the cells with 1% SDS. Optical density is therefore directly related to the number of cells attached to the substrate after the incubation time (2 hours). The data reported in the tables are the means of three different experiments in triplicate.

Table 4. Effects of LA on adhesion of murine transformed cells on laminin (10 µg/ml).

LA (µM)	MCA-1 (O.D. ± S.E.)	DBE/F4 (O.D. ± S.E.)	B(a)P (O.D. ± S.E.)
0	0.284 ± 0.020	0.286 ± 0.008	0.070 ± 0.007
100	0.262 ± 0.002	0.451 ± 0.038	0.070 ± 0.006
250	0.314 ± 0.041	0.481 ± 0.007	0.147 ± 0.004
500	0.468 ± 0.034	0.961 ± 0.116	0.173 ± 0.014

Table 5. Effects of LA on adhesion of murine transformed cells on type IV collagen (10 µg/ml).

LA (µM)	MCA-1 (O.D. ± S.E.)	DBE/F4 (O.D. ± S.E.)	B(a)P (O.D. ± S.E.)
0	0.121 ± 0.010	0.180 ± 0.004	0.135 ± 0.022
100	0.156 ± 0.015	0.221 ± 0.014	0.251 ± 0.028
250	0.172 ± 0.009	0.237 ± 0.035	0.299 ± 0.025
500	0.328 ± 0.001	0.473 ± 0.09	0.575 ± 0.026

Table 6. Effects of LA on adhesion of murine transformed cells on fibronectin (3 μg/ml)

LA (μM)	MCA-1 (O.D. ± S.E.)	DBE/F4 (O.D. ± S.E.)	B(a)P (O.D. ± S.E.)
0	1.115 ± 0.035	0.559 ± 0.048	0.476 ± 0.014
100	1.176 ± 0.019	0.614 ± 0.079	0.562 ± 0.009
250	1.153 ± 0.025	0.734 ± 0.048	0.561 ± 0.027
500	1.344 ± 0.025	0.944 ± 0.010	0.728 ± 0.004

Table 7. Effects of LA on adhesion of murine transformed cells on vitronectin (3 μg/ml)

LA (μM)	MCA-1 (O.D. ± S.E.)	DBE/F4 (O.D. ± S.E.)	B(a)P (O.D. ± S.E.)
0	1.658 ± 0.045	1.400 ± 0.053	0.877 ± 0.016
100	1.630 ± 0.100	1.434 ± 0.028	1.026 ± 0.043
250	2.292 ± 0.072	1.732 ± 0.018	1.061 ± 0.003
500	2.415 ± 0.030	2.199 ± 0.042	1.082 ± 0.043

RESULTS OF THE ADHESION ASSAY

Alpha lipoic acid induces a reduction in cell migration while enhancing adhesion to the matrix. In fact, a 500 μM concentration induces an about 2.5 times increase in adhesion to laminin and to collagen IV, and an about 1.5 times increase in adhesion to fibronectin and to vitronectin.

What stated above clearly evidence that lipoic acid or the physiologically equivalents analogues thereof (salts, esters, solvates, inclusion complexes and the like) can advantageously be used for the preparation of antimetastatic drugs.

For the scheduled therapeutical uses, lipoic acid can be administered through the oral, intravenous (US 5569670), subcutaneous (WO97/10808) routes or through the other conventional administration routes (topical, inhalatory, rectal, etc.).

Because of the extremely low toxicity of lipoic acid, it can therefore be administered at very high doses.

The possibility to carry out chronic oral administrations is of course a remarkable advantage of the present invention.

As a rule, daily posology will range from about 0.5 to about 5 g, optionally subdivided in repeated administrations, depending on the disease and the conditions of the patient (weight, sex and age).

Suitable formulations of lipoic acid can be prepared according to conventional techniques.